A Perspective

Engineering Periosteum for Structural Bone Graft Healing

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Abstract Autograft is superior to both allograft and synthetic bone graft in repair of large structural bone defect largely due to the presence of multipotent mesenchymal stem cells in periosteum. Recent studies have provided further evidence that activation, expansion and differentiation of the donor periosteal progenitor cells are essential for the initiation of osteogenesis and angiogenesis of donor bone graft healing. The formation of donor cell-derived periosteal callus enables efficient host-dependent graft repair and remodeling at the later stage of healing. Removal of periosteum from bone autograft markedly impairs healing whereas engraftment of multipotent mesenchymal stem cells on bone allograft improves healing and graft incorporation. These studies provide rationale for fabrication of a biomimetic periosteum substitute that could fit bone of any size and shape for enhanced allograft healing and repair. The success of such an approach will depend on further understanding of the molecular signals that control inflammation, cellular recruitment as well as mesenchymal stem cell differentiation and expansion

during the early phase of the repair process. It will also depend on multidisciplinary collaborations between biologists, material scientists and bioengineers to address issues of material selection and modification, biological and biomechanical parameters for functional evaluation of bone allograft healing.

Introduction

Structural bone grafting surgeries have been widely used to repair or replace bone defects caused by trauma, tumor resection, pathological degeneration, and congenital deformations. The most commonly used clinical bone grafts include autograft, allograft, and synthetic materials. Live autograft has been the gold standard for repair of large bone defects. Autograft has the ability to stimulate new bone formation by recruitment of mesenchymal stem cells from the host bed (osteoiduction). Autograft can also regenerate itself by production of new bone (osteogenesis). In contrast to an autograft, processed allograft and synthetic bone graft substitutes lack living cells. Therefore, they only function as an osteoconductive scaffold for bone ingrowth [12, 13]. For simplicity, in this article we refer to autograft as vitalized bone graft isolated from the same animal, whereas allograft is referred to as devitalized bone graft isolated from genetically different animals.

Clinical and experimental data demonstrate periosteum plays a pivotal role in bone autograft healing and remodeling [24, 47, 49, 85]. Periosteum contains multipotent mesenchymal stem cells that are capable of differentiating into bone and cartilage. However, due to the absence of proper cellular markers for this specific population of stem cells, the cellular and molecular mechanisms underlying periosteum-mediated bone repair remain largely elusive.

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Understanding the temporal and spatial control of periosteum-mediated bone repair is vital for devising novel therapies aimed at improving bone graft healing and repair.

Studies using animal models, particularly genetically modified animals, have provided novel information on control of the periosteal progenitor cell differentiation and expansion during bone graft healing. These studies are designed to specifically address questions related to the cellular contribution of progenitor cells to graft healing and the molecular signals critical for initiation of periosteal stem cell-dependent repair. Based on these studies, cellular signals that control inflammation, mesenchymal stem cell differentiation and expansion likely play key roles in periosteum initiated healing. In this article, we review recent progress made toward an understanding of the cellular mechanisms of periosteum-mediated bone graft healing. We also discuss a tissue engineering approach for fabrication of a replacement periosteum with angiogenic and osteogenic properties for enhanced structural bone allograft healing.

Search Strategy and Criteria

To identify relevant literature, we used a sensitive search of the most common databases for published medical literature, including Ovid MEDLINE[®] and PubMed. The search strings used were periosteum, structural bone graft, cortical bone graft, allograft, and autograft. We made no restrictions on date but focus on the most recent relevant publication on bone graft biology in orthopaedic related studies.

Biology of Bone Graft Incorporation and Remodeling

The repair and incorporation of bone graft is a regulated process that moves through several distinct, yet overlapping stages. The early stage of graft healing is characterized by hematoma formation, early vascular invasion, and inflammatory response, which facilitate activation and recruitment of mesenchymal stem cells [12, 13]. The expansion and differentiation of the mesenchymal stem cells in response to growth factors and cytokines at the injury site eventually result in endochondral and intramembraneous bone formation, leading to osseointegration of the graft to host bone. The later phase of graft healing is characterized by graft repair and remodeling, a process that is different between cortical bone and cancellous bone [12, 13, 27, 29, 85]. Vascularization of cancellous bone graft occurs quickly due to its porous nature and can be rapidly incorporated and remodeled. In this process, osteoblasts first produce osteoid on the surface of necrotic bone, which is subsequently

removed by osteoclasts. This process gradually resorbs the entrapped dead trabeculae and eventually replaces the entire graft with new bone. In contrast to cancellous bone, remodeling and vascularization of cortical bone are very poor and involve a process called creeping substitution. In this process, cortical bone is resorbed gradually through Haversian and Volkmann canals before replacement with new viable bone. The histologic evaluations demonstrate the repair of cortical bone graft initiates from the host-graft junction and gradually spreads to the midshaft of the structural graft. The remodeling of cortical bone occurs at a very slow rate and the dead cortical bone typically cannot be completely replaced by new bone [6, 12–14].

The outcome of structural bone graft healing is affected by several critical factors. The healing and the extent of bone graft incorporation are positively correlated with the viability of the donor graft and the vascularity of the graft bed but negatively correlated with donor-host histocompatibility antigen disparity [86]. Live autograft healing is vastly superior to processed bone graft largely due to the presence of living cells on the bone graft. Early studies using a free subcutaneous live isograft radioactively labeled with ⁸⁵SrCl₂ demonstrated free marrow cells and osteocytes have little or no contribution to early osteogenesis of bone graft, while living cells in periosteum and endosteum plus stromal cells are responsible for 90% of the early osteogenesis [30]. Remodeling and revascularization of structural bone graft are also affected by the viability of the graft and host-donor immunocompatibility. Radiographic and histologic findings of retrieved massive human bone allografts demonstrate the repair of cortical allograft occurs at an extremely slow rate, in which the newly formed bone only penetrates a few millimeters into the necrotic bone [22, 23, 92]. This limited bone formation and repair activity is directly associated with a near 20% failure rate of structural allografts due to nonunion and early fracture [8, 26, 54]. Fractures typically occur 1 to 2 years after implantation and are related to the propagation of microdamage within the dead cortical bone [95].

Periosteum is Essential for the Initiation of Bone Graft Healing and Remodeling

Periosteum consists of microvascularized connective tissue that covers the outer surface of cortical bone. Periosteum can be separated into two distinct layers: an outer layer that contains fibroblasts and Sharpey's fibers and an inner layer called cambium, which contains multipotent mesenchymal stem cells and osteoprogenitor cells that contribute to normal bone growth, healing, and regeneration [2, 5, 41, 68, 97]. Similar to bone marrow stromal cells, in

monolayer culture adherent periosteal cells demonstrate fibroblastlike morphology and express neither the osteogenic nor the chondrogenic phenotype. When inoculated heterotopically into nude mice, these cells eventually give rise to bone and cartilage tissues at the subcutaneous injection site [61–63].

To gain an understanding of the cellular and molecular mechanisms that define the superior healing of live bone autograft over allograft, we recently developed a murine segmental bone graft model that permits quantitative analyses of structural bone graft healing, remodeling, and graft-host interactions [91]. This model involves the transplantation of a 4-mm mid-diaphyseal femoral bone segment to a host with the same size defect in the femur. By comparing live isograft healing with devitalized allograft, devitalized isograft, or periosteum-free isograft, we demonstrated live periosteum is essential for bone graft healing and remodeling. Removal of periosteum from a

live bone graft resulted in a 73% decrease in new bone and cartilage formation on the graft and a 10-fold decrease in neovascularization as measured by histomorphometry and micro-CT [102]. The absence of periosteum also resulted in 75% reduction of osteoclast numbers on bone graft, which correlated with the poor remodeling activity of the devitalized bone allograft or isograft. In contrast, removing cells from the bone marrow had minimal effects on the periosteal bone formation on the donor bone graft [91].

The establishment of a murine bone-grafting model further allows us to study graft repair in genetically defined (transgenic and knockout) animals. By transplanting live bone graft derived from LacZ reporter mice (R26A) that constitutively express β -galactosidase (β -gal) in nearly all cells, we marked the early periosteal progenitors and followed the fate of these cells during the process of graft healing [102]. We found β -gal-positive cells largely localized to the region directly overlying the graft (Fig. 1A–C).

Fig. 1A–C Live donor isografts from R26A mice were transplanted into wild type β -gal negative mouse femurs. (A) H&E/alcian blue staining (original magnification at 4X) demonstrates live Rosa 26 isograft in a wild type donor produces robust endochondral and intramembranous bone formation at the cortical bone junction. Host side and graft side at the junctions are marked as * (host) and # (graft). (B) X-gal staining of adjacent sections demonstrates the majority of cartilage and bone overlying the donor graft stained positive for β -gal (blue), indicating donor origin. (C) Magnified images (20X) of the boxed region in B demonstrated nearly all the osteocytes (arrow) in newly formed bone overlying donor bone graft stained β -gal positive (blue), indicating donor origin. (A1–A5) Photomicrographs (original magnification 20X) of X-Gal-stained sections show the progression of donor periosteal progenitor cells (blue) through the endochondral bone formation from Day 1 to Day 10. (B1–B5) X-Gal (blue) and alkaline phosphatase (AP) (red) double staining demonstrate the differentiation of β -gal-positive cells from Day 1 to Day 10.

At Day 1, a layer of β -gal-positive cells that weakly stained for alkaline phosphatase (ALP) was detected on the surface of donor bone graft (Fig. 1A1, B1). At Days 3 and 5, we observed cellular condensations consisting of only donor β -gal-positive cells on the surface of the donor graft (Fig. 1A2–A3). Most of these cells were ALP-negative (Fig. 1B2–B3) but bromodeoxyuridine-positive (not shown). At Day 7, hypertrophic chondrocytes were qualitatively highly positive for ALP staining, whereas a large proportion of β -gal-positive surrounding mesenchymal cells remained low or negative for ALP (Fig. 1A4, B4). At Day 10, β -gal-positive mesenchymal progenitor cells were completely replaced by β -gal-positive, ALP-positive hypertrophic chondrocytes (Fig. 1A5, B5). Donor cells eventually gave rise to chondrocytes, osteocytes, osteoblasts, and vessel-lining cells demonstrating the pluripotent nature of stem cells residing in the periosteum.

The marking of donor progenitor cells further permits quantitative analysis of donor cell contribution to bone repair. Histomorphometric analyses were performed to determine the total bone and cartilage formation on the host and donor graft sides. Bone and cartilage derived from β -gal-positive donor progenitor cells were also measured. Our data demonstrated, on Days 7 and 10, 70–90% of cartilaginous tissue and new woven bone on the graft side was positive for β -gal. However, after Day 10, host-dependent remodeling of cartilage and new bone resulted in rapid replacement of donor tissues and cells, such that by Day 28, few osteoblasts or osteocytes were positive for β -gal.

The marking of the dynamic progression of donor periosteal reparative callus formation suggested live bone iso- or autograft healing was initiated and driven by pluripotent local mesenchymal stem cells [102]. The initiation of graft repair required activation, differentiation, and expansion of residing periosteal stem/progenitor cells. The differentiation of stem/periosteal cells induced robust chondrogenesis and osteogenesis, which served as essential templates for host cell invasion. On the other hand, our data also suggest the completion of bone repair and remodeling was a completely host-dependent process, which required blood vessels to bring in host osteoblast and osteoclast progenitor cells to further repair and remodel bone graft. The dynamic remodeling of donor bone graft was also demonstrated in a vascularized bone isograft transplantation model in rat [59, 60].

Molecular Signals Control the Morphogenesis of Periosteum during Healing

The molecular signals controlling the initiation and morphogenesis of periosteal bone repair are only superficially

understood. Experimental data suggest periosteum-initiated bone repair may be analogous to fetal limb bud development [25], which capitulates the fundamental features of bone and cartilage differentiation in the developing limb [77]. In the past decade, progress has been made to identify factors and genes involved in morphogenesis of the limb bud. The most notable ones are the bone morphogenic proteins (BMPs) that belong to the transforming growth factor beta superfamily, Hedgehog proteins, and Wnt proteins. Others include fibroblast growth factors (FGFs) and insulinlike growth factors (IGFs), which have positive effects on bone healing [44, 55, 83]. BMP-2 expression occurred in early periosteal callus just a few days after cortical bone fracture [11]. Most recently, Tsuji et al. [93] demonstrated elimination of BMP-2 in the limb does not affect the limb development but disrupts the initiation of postnatal fracture healing. Evidence has also emerged to demonstrate morphogens such as Hhgs and Wnt proteins, which are involved in embryonic pattern formation, also function postembryonically to initiate pathways that control self-renewal, migration, differentiation, and cell fate commitment of adult stem or progenitor cells [7, 57]. Detailed studies are needed to determine the role of these two pathways in regulation of the morphogenesis of adult bone healing.

In addition to mechanisms analogous to limb development, genes involved in injury and inflammatory responses during the repair process also play key roles in bone graft healing and periosteal endochondral bone formation. Nonsteroidal antiinflammatory drugs (NSAIDs) are the most commonly used pain-relieving medications in our society. NSAIDs inhibit cyclooxygenase (COX), which catalyzes the formation of prostaglandins and thromboxanes from arachidonic acid [94]. It is now known at least two isoforms of COX exist, the inducible isoform COX-2 and the constitutive isoform COX-1 [37]. Several studies report COX activity is involved in normal bone metabolism and suggest NSAIDs have a negative impact on bone repair [39, 82, 88]. The most compelling data implicating COX activity during bone repair come from genetic models that demonstrate a critical role for COX-2. Work in our laboratory [101] and that of others [82] documents defective periosteal bone repair in COX-2^{-/-} mice after fracture. To better understand the role of COX-2 inhibition, in a recent study we used a gain- and loss-of-function approach in the murine segmental femur allograft-healing model [91]. We observed an inhibitory effect of a selective COX-2 inhibitor celecoxib at a dose of 25 mg/kg on bone formation and graft incorporation. The inhibitory effect remained after withdrawal of short-term administration of celecoxib for the initial 2 weeks. Since PGE₂, the major metabolites from the activity of COX, is induced during the early inflammatory phase of healing, we infused PGE₂ locally at

the cortical bone junction via osmotic minipumps. We demonstrated constitutive administration of prostaglandin E2 for 2 weeks increased allograft healing via increasing host bone formation at the allograft junction [67].

Functional Tissue Engineering of Periosteum for Bone Graft Healing and Repair

Tissue engineering holds great promise for tissue repair and regeneration [10, 16, 31]. While reconstruction of small- to moderate-sized bone defects via tissue engineering is technically feasible, the reconstruction of large load-bearing defects remains challenging. So far, few of the hard tissue scaffolds have been used clinically for repair of large defects [15]. On the other hand, massive allografts can restore the size and shape of the resected bone. Compared to hard tissue scaffolds, such as ceramics, structural bone allografts possess superior mechanical strength and fracture resistance and therefore remain a better choice for repair of large defects that require immediate support [18, 90]. However, due to the lack of viable angiogenic and osteogenic cells on the graft, the healing and remodeling of bone allograft is extremely limited. Based on the studies described above, which demonstrated the essential role of the periosteum in the initiation of autograft healing, we reasoned inferior healing of bone allograft is largely due to the lack of osteogenic and angiogenic periosteum on the graft. To restore the essential osteogenic and angiogenic activity of periosteum on bone allograft, we devised an approach by engrafting mesenchymal stem cells engineered to express the osteogenic and angiogenic factor BMP-2 around bone allograft. This approach led to early induction of a reparative response, increase in angiogenesis, and the formation of a cortical shell around the grafted allobone. As a result, the incorporation of the allograft was enhanced and the inferior biomechanical performance of the allografted femurs was reversed [98, 102].

Revitalizing acellular surfaces with angiogenic and osteogenic mesenchymal stem cells that mimic a periosteal response during initiation of repair may have broad applications. Several recent papers have illustrated an approach using mesenchymal stem cell sheets wrapping around bone graft or synthetic graft materials for enhanced healing. Zhou et al. [103] assembled cell sheets comprising multilayered porcine bone marrow stromal cells with polycaprolactone-calcium phosphate for the engineering of structural and functional bone grafts. The layered cell sheets integrated into the scaffold/cell construct. *in vivo*, cell-sheet-scaffold/cell constructs formed bone via predominantly endochondral ossification when implanted under the skin of nude rats [103]. Ouyang et al. [69] also reported a similar approach of using mesenchymal stem

cell sheets to revitalize nonviable dense grafts and tendon. Using an ovine femoral model, Knothe Tate et al. [48] used an *in situ* periosteal sleeve that was elevated circumferentially from diaphyseal bone to wrap around cortical bone chips. They reported the periosteal sleeve-combined autograft bone chips were most effective in repair of critical-sized defects in the femur [48].

Design Considerations for Functional Tissue Engineering of Periosteum

Based on the experimental data obtained from animal models, we propose the application of tissue-engineering principles to develop a flexible cellular construct that serves as an osteogenic and angiogenic “periosteum” to fit around bone or bone substitute of any size or shape for enhanced bone repair and regeneration. Current advances in stem cell biology, gene therapy, and biomaterial fabrication enable the biomimetic design of such a cellular construct suitable for structural bone graft application. To successfully generate such a cellular scaffold, three important components must be considered: (1) live osteogenic cells that are capable of producing new bone; (2) osteoinductive genes or factors; and (3) an osteoconductive scaffold.

The most commonly used osteogenic cells are bone marrow stromal cells derived from bone marrow aspirates. These cells can be easily expanded in culture. When inoculated heterotopically into nude mice, these cells eventually give rise to bone and cartilage tissues [9]. Other sources of cells used in bone tissue engineering include adipose-derived stem cells [32] and periosteal cells [40, 65]. Human dermal fibroblasts are another attractive source of cells that can meet the criteria for bone tissue engineering [89]. Recent studies have investigated the ability of cells committed to a fibroblastic lineage to undergo osteogenic differentiation. Specifically, human gingival and dermal fibroblasts were able to express osteoblast differentiation markers after transduction with vectors overexpressing BMPs [38, 50, 76]. Human dermal fibroblasts cultured on ultraporous β -tricalcium phosphate ceramics demonstrated increased osteogenic differentiation [36]. Phillips et al. [73, 74] reported primary dermal fibroblasts overexpressing Runx2 can be used as a mineralizing cell source from bone tissue engineering.

BMPs have been widely used as osteoinductive factor for bone tissue repair and regeneration. Although successful in animal models, the effectiveness of BMPs in humans requires large doses and sustained delivery of active proteins. Furthermore, BMP-2 seems to have limited effects on nonunions that mostly consist of fibroblastic tissues. In one such application [20], two allograft fracture

nonunions and one nonunion at the allograft-host junction were treated with 12 mg rhBMP-2. The remaining three nonunions were treated with 7 mg rhBMP-7 (Osigraft[®]; Stryker Biotech, Hopkinton, MA). The outcome and radiographic evidence of healing were evaluated at a minimal followup of 12 months. There was neither healing of allograft fractures nor union of allograft-host junction [20]. Thus, improving the efficacy of BMP treatment and searching for alternative osteogenic factors or genes have become the most important topics in bone regeneration therapy. Cooperation of two or multiple pathways to promote bone formation in a tissue-engineering application has not yet been fully explored and is fertile ground for future investigation [58, 99].

Recent studies suggest intermittent administration of parathyroid hormone (PTH) enhances bone healing and repair. Specifically administration of the amino terminal fragment PTH 1–34 demonstrated stimulatory effects on healing in various animal models including fracture-healing models [3, 45, 66, 96] and bone-grafting models [1, 35]. The mechanism by which PTH stimulates bone healing is not well understood. Several recent studies suggest PTH exerts its anabolic actions at both the early stage and the late stage of bone healing [3, 64]. Intermittent treatment of PTH altered the expression of IGFs and Wnt signaling in fracture-healing models [43, 66].

Several studies demonstrate adult stem/progenitor cells reside in a unique microenvironment composed of extracellular matrix and the resident cells (collectively called a niche) [19, 100]. It is likely assembly or modification of such a microenvironment may direct lineage-specific differentiation of stem/progenitor cells, thereby producing specific cells or tissues for regeneration purposes. It is for this reason synthesis of nanofibers to mimic the architecture of tissue-specific extracellular matrix has drawn a great deal of attention. Currently, there are three techniques available for the synthesis of nanofibers: electrospinning, self-assembly, and phase separation [42, 56]. Of these techniques, electrospinning has emerged as a simple and versatile technique that can produce a porous scaffold comprising randomly oriented or aligned nanofibers characteristic of extracellular matrix. Combined with various design polymers, fibers can incorporate drug delivery function and biochemical signals into the scaffold [4]. Endowed with both topographic and biochemical signals, such nanofibrous scaffolds may provide an optimal microenvironment for the seeded cells [46].

Studies using nanofibers for bone tissue-engineering applications also indicate the nanofibers promote bone cell differentiation. Several such scaffolds have demonstrated increased cellular infiltration and decreased fibrotic response compared to conventional scaffolds [71, 80]. Most recently, three-dimensional (3-D) tissue fabrication

via electrospinning of matrices and concurrent electrospraying of precursor cells have been reported [84]. In this application, nanofibers of a biodegradable polymer, elastomeric poly(ester urethane)urea, were spun together with vascular smooth muscle cells. The resulting 3-D cell-fiber constructs demonstrated satisfactory tensile strength, as well as growth and proliferation of progenitor cells within the fibers, demonstrating the great potential and versatility of this technique in 3-D tissue fabrications.

Design Rationale and Outcome Evaluations for Tissue-engineered Cellular Constructs for Bone Allograft Healing

To evaluate the functional performance of the biomimetic cellular scaffold for bone graft healing, the following parameters should be considered: (1) osseointegration of allograft to host bone; (2) neovascularization of the graft; (3) remodeling of the graft; and (4) biomechanics of bone allograft. These parameters constitute important benchmarks for evaluation of efficacy for bone graft incorporation and repair.

Due to the lack of living cells on bone allograft, the osseointegration of allograft to host bone is slow and limited. In addition, the absence of osteogenic and angiogenic activity of the necrotic cortical bone often cause persistent fibrotic tissue formation. Thus, the primary goal of the cell-based therapy for bone allograft healing is to inhibit fibrotic tissue formation and accelerate osseointegration or incorporation of allograft to host bone. Osseointegration between donor graft and host bone is a challenging problem in graft repair. If not properly guided, newly formed bone could be poorly organized and therefore contribute little to the biomechanical function of the graft. A successful cellular replacement for periosteum should be designed to offer the directional bone growth, eg, to inhibit fibrotic tissue formation while promoting differentiation, proliferation, and directional migration of osteoblastic cells along bone allograft surface. Several innovative approaches have been used to add appropriate surface topographies to membranes in guided tissue regeneration for periodontal tissue regeneration [70, 81]. Conceivably, these techniques could also be used in our current design for regeneration of periosteal bone formation.

Experimental and clinical experience indicate vascularity of the graft bed is critical for bone graft healing and incorporation [86]. Vascularity is essential for providing optimal blood supply to maintain survival of the osteogenic cells. The angiogenic response from the host is five to 10 times greater with fresh isograft compared to cryopreserved isograft in the subcutaneous bone graft transplantation model [52, 53]. A

reciprocal role of angiogenesis and osteogenesis in bone repair has been illustrated in several bone repair and regeneration models [28, 87]. Bone formation can be enhanced by angiogenic growth factors such as vascular endothelial growth factor (VEGF), FGF, or platelet-derived growth factor (PDGF). VEGF further synergizes with BMP-2 stimulating bone formation [72]. In contrast, suppression of angiogenesis blocks bone formation. On the other hand, osteogenic factors, including BMPs, are potent stimulators for angiogenesis, suggesting bone formation depends on angiogenesis.

To evaluate the neovascularization during bone graft healing, we have developed micro-CT image-based 3D stereologic techniques for quantifying the vasculature and new bone callus formation for structural graft healing [102]. This approach allows analysis of bone and vasculature in the same sample. When applied to a direct model for the analysis of vasculature, we can perform histomorphometric analysis to determine the vascular volume, connectivity, vessel number, mean vessel thickness, vessel separation, and degree of anisotropy in a femoral bone-grafting model. This approach ensures accurate nondestructive evaluation of structural bone graft healing and vascularization [21, 33, 34, 102].

Due to the limited osteogenesis and poor vascularity of devitalized bone, remodeling of structural bone graft is limited. In some cases, decoupling of graft remodeling occurs due to insufficient osteoblast recruitment and activity, which results in loss of bone stock and failure of the grafted allograft bone [22, 23]. The only effective way to save the failing allograft is to place a vascularized autograft side by side with the allograft. The transplantation of an autograft improves vascularity and brings osteogenic periosteal cells to repair the failing allograft. The data obtained from retrieved large human bone allograft suggest the remodeling of the allograft is positively correlated with the extent of osteogenesis on the graft. The area near the host-graft junction is remodeled more extensively than the area at the midshaft of the allograft. Based on these findings, we presume enhanced osteogenesis and angiogenesis on bone allograft will facilitate bone allograft remodeling and curb early osteoclastic bone loss. In our subsequent studies, in which we implanted BMP-2-producing mesenchymal stem cells around bone allograft [98], we observed that with improved vascularity and enhanced bone formation surrounding allografted bone the remodeling of necrotic bone allograft was improved compared to acellular allograft. However, the extent of resorption and remodeling was slower than autograft, in which the donor graft was rapidly replaced by new bone 9 weeks after transplantation [98, 102]. With reduced resorption and improved incorporation of the allograft, we found transplantation of stem cell treated allograft resulted

in better biomechanics, outperforming live bone isograft in torsional testing [98]. The delayed resorption of bone allograft could be due to the lack of living cells in bone, eg, osteocytes.

Evaluation of mechanical performance of the allografted bone is essential for determining the outcome of allograft healing. Mechanical analyses also provide the opportunity to study the structure-function relationship in bone graft healing if combined with micro-CT-based imaging analyses. Identification of micro-CT-based geometric parameters that correlate to the mechanical properties of the healing bone may be useful to interpret the effects of novel therapeutic interventions [78, 79]. Once established, these parameters can be used to evaluate and predict the functional performance of clinical bone graft using noninvasive imaging technology. Using a murine bone-grafting model, we demonstrated that although allograft incorporated into the host over time, the mechanical properties remain severely impaired compared to live autografts mainly due to the lack of new bone around donor bone graft. Unlike autografts that have a bridging callus by 6 weeks, allografts generate a callus that remains limited to the host side at the cortical bone junction at 6 weeks, and slowly creeps onto the allograft cortex and remodels down thereafter. An analysis of the connectivity of the graft to the host bones indicates that allografts are largely disconnected from the host especially at 6 weeks. From these studies, we identified three predictive correlations between combinations of the micro-CT parameters and the measured ultimate torque and torsional rigidity of the murine grafts, respectively [75, 98]: (1) amount of new bone formation around bone graft; (2) cross-sectional polar moment of inertia of the graft callus; and (3) connectivity of the bone allograft to the host bone.

Discussion

Studies using genetically modified animal models in bone graft transplantation demonstrated the essential role of periosteal stem/progenitor cells in the initiation of bone graft healing and repair. These studies provide further rationale for revitalization of bone allograft for reconstruction of large segmental defects using osteogenic and angiogenic stem cells. Although most of the studies cited in this article were conducted in small animal models such as mouse and rat that have limitations in their interpretation for human conditions, these studies have been particularly instrumental in dissection of cellular and molecular mechanisms for the biology of bone grafting.

We note several limitations. Our review was not systematic and rather selective; we did track the numbers of articles we identified with each search strategy and we had

no specific selection criteria. The lack of osteogenic and angiogenic property of a structural bone allograft has severely restricted its use in orthopaedic reconstructive surgeries. Fabrication of a biomimetic cellular scaffold that mimics the osteogenic and angiogenic function of periosteum may provide an effective approach to treat patients with compromised periosteum at risk of nonunion. Toward this end, a multidisciplinary strategy in collaboration with stem cell biologists, bioengineers, and biomaterial scientists as well as clinicians is of central importance.

For bone biologists, the future challenge is a deeper understanding of the cellular and molecular biology of periosteum in bone graft healing and repair. A biomimetic strategy will have to be built upon a thorough understanding of the function and behavior of each cellular component residing in periosteum, most notably the functional relationship of vascularization and bone formation. Inspiration may be drawn from the genetic blueprint of limb development in which mouse genetics has offered unlimited power for mapping the temporal and spatial regulation of bone and cartilage formation. Future studies to identify the essential signals or signaling pathways that control periosteal stem/progenitor cell activation, differentiation, and expansion during repair are important since these studies will provide important rationale for the timing and concentrations of osteogenic and angiogenic factors and cytokines fabricated on the periosteal constructs. In addition, maximizing the host contribution to bone graft healing and stimulating host stem cell homing to repair site will help design a “self-propelled” engineering construct for effective repair and remodeling.

For biomaterial scientists, the challenge will rest on the innovative material design and fabrication that mimic the structural and topographic cues of the healing periosteum at a micrometer or nanometer scale. The future matrices for biomimetic design must incorporate peptide or protein motifs to target adhesion, proliferation, and differentiation of mesenchymal stem cells that mediate the earlier stages of bone repair. In addition, it will need to incorporate drug delivery function and possibly the mechanical features that facilitate or augment osseointegration and remodeling of the bone graft. Finding cost effective, biocompatible, bioresorptive, noninflammatory materials that mimic the fast turnover of periosteum will be essential for success of this application.

It is known from clinical practice and animal experimentation biomechanical factors influence the outcome of bone graft repair and remodeling [86]. Mechanical instability could affect the differentiation and proliferation of multipotent mesenchymal stem cells during repair and regeneration [17, 51]. In addition, loading and weight-bearing affect new bone callus remodeling during bone healing. The mechanisms by which mechanical stimuli

direct rapid remodeling of autograft are not fully understood. Understanding the role of biomechanics during initiation and remodeling of bone graft healing is important for rational design of cellular constructs that mimic the biologic and biomechanical function of the periosteum.

Mechanisms that control periosteum-initiated bone graft healing remain poorly understood. Engineering of an effective cellular replacement for allograft healing requires a deeper understanding of the molecular pathways that control differentiation and expansion of periosteal progenitor cells. It is also absolutely critical to acquire further knowledge on the biology and biomechanics of bone graft incorporation and remodeling. With exciting current advances in stem cell biology, genetics, tissue engineering, gene therapy, matrix synthesis, and nanotechnology, fabrication of such an angiogenic and osteogenic cellular construct as a replacement for periosteum will undoubtedly improve the usage of structural allograft. The development of a flexible, biomimetic osteogenic cellular scaffold will also have broad application in orthopaedic reconstruction.

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References

1. Abe Y, Takahata M, Ito M, Irie K, Abumi K, Minami A. Enhancement of graft bone healing by intermittent administration of human parathyroid hormone (1–34) in a rat spinal arthrodesis model. *Bone*. 2007;41:775–785.
2. Allen MR, Hock JM, Burr DB. Periosteum: biology, regulation, and response to osteoporosis therapies. *Bone*. 2004; 35:1003–1012.
3. Andreassen TT, Ejersted C, Oxlund H. Intermittent parathyroid hormone (1–34) treatment increases callus formation and mechanical strength of healing rat fractures. *J Bone Miner Res*. 1999;14:960–968.
4. Ashammakhi N, Ndreu A, Piras A, Nikkila L, Sindelar T, Ylikauppila H, Harlin A, Chiellini E, Hasirci V, Redl H. Biodegradable nanomats produced by electrospinning: expanding multifunctionality and potential for tissue engineering. *J Nanosci Nanotechnol*. 2006;6:2693–2711.
5. Augustin G, Antabak A, Davila S. The periosteum Part 1: Anatomy, histology and molecular biology. *Injury*. 2007;38: 1115–1130.
6. Bauer TW, Muschler GF. Bone graft materials. An overview of the basic science. *Clin Orthop Relat Res*. 2000;371:10–27.
7. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. *Nature*. 2004;432:324–331.
8. Berrey BH Jr, Lord CF, Gebhardt MC, Mankin HJ. Fractures of allografts. Frequency, treatment, and end-results. *J Bone Joint Surg Am*. 1990;72:825–833.
9. Bianco P, Rinnucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells*. 2001;19:180–192.
10. Bianco P, Robey PG. Stem cells in tissue engineering. *Nature*. 2001;414:118–121.
11. Bostrom MP, Lane JM, Berberian WS, Missri AA, Tomin E, Weiland A, Doty SB, Glaser D, Rosen VM. Immunolocalization

- and expression of bone morphogenetic proteins 2 and 4 in fracture healing. *J Orthop Res.* 1995;13:357–367.
12. Burchardt H. The biology of bone graft repair. *Clin Orthop Relat Res.* 1983;174:28–42.
 13. Burchardt H. Biology of bone transplantation. *Orthop Clin North Am.* 1987;18:187–196.
 14. Burchardt H, Enneking WF. Transplantation of bone. *Surg Clin North Am.* 1978;58:403–427.
 15. Cancedda R, Giannoni P, Mastrogiacomo M. A tissue engineering approach to bone repair in large animal models and in clinical practice. *Biomaterials.* 2007;28:4240–4250.
 16. Caplan AI. in vivo remodeling. *Ann NY Acad Sci.* 2002;961:307–308.
 17. Carter DR, Beaupre GS, Giori NJ, Helms JA. Mechanobiology of skeletal regeneration. *Clin Orthop Relat Res.* 1998;355 Suppl:S41–55.
 18. Davy DT. Biomechanical issues in bone transplantation. *Orthop Clin North Am.* 1999;30:553–563.
 19. Dawson E, Mapili G, Erickson K, Taqvi S, Roy K. Biomaterials for stem cell differentiation. *Adv Drug Deliv Rev.* 2008;60:215–228.
 20. Delloye C, Simon P, Nyssen-Behets C, Banse X, Bresler F, Schmitt D. Perforations of cortical bone allografts improve their incorporation. *Clin Orthop Relat Res.* 2002;396:240–247.
 21. Duvall CL, Robert Taylor W, Weiss D, Guldberg RE. Quantitative microcomputed tomography analysis of collateral vessel development after ischemic injury. *Am J Physiol Heart Circ Physiol.* 2004;287:H302–310.
 22. Enneking WF, Campanacci DA. Retrieved human allografts: a clinicopathological study. *J Bone Joint Surg Am.* 2001;83:971–986.
 23. Enneking WF, Mindell ER. Observations on massive retrieved human allografts. *J Bone Joint Surg Am.* 1991;73:1123–1142.
 24. Eyre-Brook AL. The periosteum: its function reassessed. *Clin Orthop Relat Res.* 1984;189:300–307.
 25. Ferguson C, Alpern E, Miclau T, Helms JA. Does adult fracture repair recapitulate embryonic skeletal formation? *Mech Dev.* 1999;87:57–66.
 26. Fox EJ, Hau MA, Gebhardt MC, Hornicek FJ, Tomford WW, Mankin HJ. Long-term followup of proximal femoral allografts. *Clin Orthop Relat Res.* 2002;397:106–113.
 27. Garbuz DS, Masri BA, Czitrom AA. Biology of allografting. *Orthop Clin North Am.* 1998;29:199–204.
 28. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med.* 1999;5:623–628.
 29. Goldberg VM, Stevenson S. The biology of bone grafts. *Semin Arthroplasty.* 1993;4:58–63.
 30. Gray JC, Elves MW. Donor cells' contribution to osteogenesis in experimental cancellous bone grafts. *Clin Orthop Relat Res.* 1982;163:261–271.
 31. Griffith LG, Naughton G. Tissue engineering—current challenges and expanding opportunities. *Science.* 2002;295:1009–1014.
 32. Guilak F, Awad HA, Fermor B, Leddy HA, Gimple JM. Adipose-derived adult stem cells for cartilage tissue engineering. *Biorheology.* 2004;41:389–399.
 33. Guldberg RE, Ballock RT, Boyan BD, Duvall CL, Lin AS, Nagaraja S, Oest M, Phillips J, Porter BD, Robertson G, Taylor WR. Analyzing bone, blood vessels, and biomaterials with microcomputed tomography. *IEEE Eng Med Biol Mag.* 2003;22:77–83.
 34. Guldberg RE, Oest M, Lin AS, Ito H, Chao X, Gromov K, Goater JJ, Koefoed M, Schwarz EM, O'Keefe RJ, Zhang X. Functional integration of tissue-engineered bone constructs. *J Musculoskelet Neuronal Interact.* 2004;4:399–400.
 35. Hashimoto T, Shigetomi M, Ohno T, Matsunaga T, Muramatsu K, Tanaka H, Sugiyama T, Taguchi T. Sequential treatment with intermittent low-dose human parathyroid hormone (1–34) and bisphosphonate enhances large-size skeletal reconstruction by vascularized bone transplantation. *Calcif Tissue Int.* 2007;81:232–239.
 36. Hee CK, Jonikas MA, Nicoll SB. Influence of three-dimensional scaffold on the expression of osteogenic differentiation markers by human dermal fibroblasts. *Biomaterials.* 2006;27:875–884.
 37. Herschman HR. Prostaglandin synthase 2. *Biochem. Biophys. Acta.* 1996;1229:125–140.
 38. Hirata K, Tsukazaki T, Kadowaki A, Furukawa K, Shibata Y, Moriishi T, Okubo Y, Bessho K, Komori T, Mizuno A, Yamaguchi A. Transplantation of skin fibroblasts expressing BMP-2 promotes bone repair more effectively than those expressing Runx2. *Bone.* 2003;32:502–512.
 39. Ho ML, Chang JK, Wang GJ. Effects of ketorolac on bone repair: A radiographic study in modeled demineralized bone matrix grafted rabbits. *Pharmacology.* 1998;57:148–159.
 40. Huttmacher DW, Sittering M. Periosteal cells in bone tissue engineering. *Tissue Eng.* 2003;9 Suppl 1:S45–64.
 41. Ito Y, Fitzsimmons JS, Sanyal A, Mello MA, Mukherjee N, O'Driscoll SW. Localization of chondrocyte precursors in periosteum. *Osteoarthritis Cartilage.* 2001;9:215–223.
 42. Jayaraman K, Kotaki M, Zhang Y, Mo X, Ramakrishna S. Recent advances in polymer nanofibers. *J Nanosci Nanotechnol.* 2004;4:52–65.
 43. Kakar S, Einhorn TA, Vora S, Miara LJ, Hon G, Wigner NA, Toben D, Jacobsen KA, Al-Sebaei MO, Song M, Trackman PC, Morgan EF, Gerstenfeld LC, Barnes GL. Enhanced chondrogenesis and Wnt-signaling in parathyroid hormone treated fractures. *J Bone Miner Res.* 2007;22:1903–1912.
 44. Kandziora F, Pflugmacher R, Scholz M, Knispel C, Hiller T, Schollmeier G, Bail H, Schmidmaier G, Duda G, Raschke M, Haas NP. Comparison of BMP-2 and combined IGF-I/TGF- α 1 application in a sheep cervical spine fusion model. *Eur Spine J.* 2002;11:482–493.
 45. Kim HW, Jahng JS. Effect of intermittent administration of parathyroid hormone on fracture healing in ovariectomized rats. *Iowa Orthop J.* 1999;19:71–77.
 46. Kim TG, Park TG. Biomimicking extracellular matrix: cell adhesive RGD peptide modified electrospun poly(D,L-lactic-co-glycolic acid) nanofiber mesh. *Tissue Eng.* 2006;12:221–233.
 47. King KF. Periosteal pedicle grafting in dogs. *J Bone Joint Surg Br.* 1976;58:117–121.
 48. Knothe Tate ML, Ritzman TF, Schneider E, Knothe UR. Testing of a new one-stage bone-transport surgical procedure exploiting the periosteum for the repair of long-bone defects. *J Bone Joint Surg Am.* 2007;89:307–316.
 49. Kostopoulos L, Karring T. Role of periosteum in the formation of jaw bone. An experiment in the rat. *J Clin Periodontol.* 1995;22:247–254.
 50. Krebsbach PH, Gu K, Franceschi RT, Rutherford RB. Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone in vivo. *Hum Gene Ther.* 2000;11:1201–1210.
 51. Le AX, Miclau T, Hu D, Helms JA. Molecular aspects of healing in stabilized and non-stabilized fractures. *J Orthop Res.* 2001;19:78–84.
 52. Leunig M, Demhartner TJ, Sckell A, Fraitzl CR, Gries N, Schenk RK, Ganz R. Quantitative assessment of angiogenesis and osteogenesis after transplantation of bone: comparison of isograft and allograft bone in mice. *Acta Orthop Scand.* 1999;70:374–380.
 53. Leunig M, Yuan F, Berk DA, Gerweck LE, Jain RK. Angiogenesis and growth of isografted bone: quantitative in vivo assay in nude mice. *Lab Invest.* 1994;71:300–307.

54. Lord CF, Gebhardt MC, Tomford WW, Mankin HJ. Infection in bone allografts. Incidence, nature, and treatment. *J Bone Joint Surg Am.* 1988;70:369–376.
55. Ma T, Gutnick J, Salazar B, Larsen MD, Suenaga E, Zilber S, Huang Z, Huddleston J, Smith RL, Goodman S. Modulation of allograft incorporation by continuous infusion of growth factors over a prolonged duration in vivo. *Bone.* 2007;41:386–392.
56. Ma Z, Kotaki M, Inai R, Ramakrishna S. Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Eng.* 2005;11:101–109.
57. Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. *Neuron.* 2003;39:937–950.
58. Mbalaviele G, Sheikh S, Stains JP, Salazar VS, Cheng SL, Chen D, Civitelli R. Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. *J Cell Biochem.* 2005;94:403–418.
59. Muramatsu K, Bishop AT. Cell repopulation in vascularized bone grafts. *J Orthop Res.* 2002;20:772–778.
60. Muramatsu K, Valenzuela RG, Bishop AT. Detection of chimerism following vascularized bone allotransplantation by polymerase chain reaction using a Y-chromosome specific primer. *J Orthop Res.* 2003;21:1056–1062.
61. Nakahara H, Bruder SP, Goldberg VM, Caplan AI. in vivo osteochondrogenic potential of cultured cells derived from the periosteum. *Clin Orthop Relat Res.* 1990;259:223–232.
62. Nakahara H, Bruder SP, Haynesworth SE, Holeccek JJ, Baber MA, Goldberg VM, Caplan AI. Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. *Bone.* 1990;11:181–188.
63. Nakahara H, Goldberg VM, Caplan AI. Culture-expanded human periosteal-derived cells exhibit osteochondral potential in vivo. *J Orthop Res.* 1991;9:465–476.
64. Nakazawa T, Nakajima A, Shiomi K, Moriya H, Einhorn TA, Yamazaki M. Effects of low-dose, intermittent treatment with recombinant human parathyroid hormone (1–34) on chondrogenesis in a model of experimental fracture healing. *Bone.* 2005;37:711–719.
65. O'Driscoll SW, Fitzsimmons JS. The role of periosteum in cartilage repair. *Clin Orthop Relat Res.* 2001;391 Suppl:S190–207.
66. Okazaki K, Jingushi S, Ikenoue T, Urabe K, Sakai H, Iwamoto Y. Expression of parathyroid hormone-related peptide and insulin-like growth factor I during rat fracture healing. *J Orthop Res.* 2003;21:511–520.
67. O'Keefe RJ, Tiyapatanaputi P, Xie C, Li TF, Clark C, Zuscik MJ, Chen D, Drissi H, Schwarz E, Zhang X. COX-2 has a critical role during incorporation of structural bone allografts. *Ann NY Acad Sci.* 2006;1068:532–542.
68. Orwoll ES. Toward an expanded understanding of the role of the periosteum in skeletal health. *J Bone Miner Res.* 2003;18:949–954.
69. Ouyang HW, Cao T, Zou XH, Heng BC, Wang LL, Song XH, Huang HF. Mesenchymal stem cell sheets revitalize nonviable dense grafts: implications for repair of large-bone and tendon defects. *Transplantation.* 2006;82:170–174.
70. Owen GR, Jackson J, Chehroudi B, Burt H, Brunette DM. A PLGA membrane controlling cell behaviour for promoting tissue regeneration. *Biomaterials.* 2005;26:7447–7456.
71. Park KE, Kang HK, Lee SJ, Min BM, Park WH. Biomimetic nanofibrous scaffolds: preparation and characterization of PGA/chitin blend nanofibers. *Biomacromolecules.* 2006;7:635–643.
72. Peng H, Wright V, Usas A, Gearhart B, Shen HC, Cummins J, Huard J. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *J Clin Invest.* 2002;110:751–759.
73. Phillips JE, Guldberg RE, Garcia AJ. Dermal fibroblasts genetically modified to express Runx2/Cbfa1 as a mineralizing cell source for bone tissue engineering. *Tissue Eng.* 2007;13:2029–2040.
74. Phillips JE, Huttmacher DW, Guldberg RE, Garcia AJ. Mineralization capacity of Runx2/Cbfa1-genetically engineered fibroblasts is scaffold dependent. *Biomaterials.* 2006;27:5535–5545.
75. Reynolds DG, Hock C, Shaikh S, Jacobson J, Zhang X, Rubery PT, Beck CA, O'Keefe RJ, Lerner AL, Schwarz EM, Awad HA. Micro-computed tomography prediction of biomechanical strength in murine structural bone grafts. *J Biomech.* 2007;40:3178–3186.
76. Rutherford RB, Moalli M, Franceschi RT, Wang D, Gu K, Krebsbach PH. Bone morphogenetic protein-transduced human fibroblasts convert to osteoblasts and form bone in vivo. *Tissue Eng.* 2002;8:441–452.
77. Sandell LJ, Adler P. Developmental patterns of cartilage. *Front Biosci.* 1999;4:D731–742.
78. Shefelbine SJ, Augat P, Claes L, Simon U. Trabecular bone fracture healing simulation with finite element analysis and fuzzy logic. *J Biomech.* 2005;38:2440–2450.
79. Shefelbine SJ, Simon U, Claes L, Gold A, Gabet Y, Bab I, Muller R, Augat P. Prediction of fracture callus mechanical properties using micro-CT images and voxel-based finite element analysis. *Bone.* 2005;36:480–488.
80. Shih YR, Chen CN, Tsai SW, Wang YJ, Lee OK. Growth of mesenchymal stem cells on electrospun type I collagen nanofibers. *Stem Cells.* 2006;24:2391–2397.
81. Siebers MC, ter Brugge PJ, Walboomers XF, Jansen JA. Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. *Biomaterials.* 2005;26:137–146.
82. Simon AM, Manigrasso MB, O'Connor JP. Cyclo-oxygenase 2 function is essential for bone fracture healing. *J Bone Miner Res.* 2002;17:963–976.
83. Srouji S, Blumenfeld I, Rachmiel A, Livne E. Bone defect repair in rat tibia by TGF-beta1 and IGF-1 released from hydrogel scaffold. *Cell Tissue Bank.* 2004;5:223–230.
84. Stankus JJ, Guan J, Fujimoto K, Wagner WR. Microintegrating smooth muscle cells into a biodegradable, elastomeric fiber matrix. *Biomaterials.* 2006;27:735–744.
85. Stevenson S. Biology of bone grafts. *Orthop Clin North Am.* 1999;30:543–552.
86. Stevenson S, Emery SE, Goldberg VM. Factors affecting bone graft incorporation. *Clin Orthop Relat Res.* 1996;324:66–74.
87. Street J, Bao M, deGuzman L, Bunting S, Peale FV Jr, Ferrara N, Steinmetz H, Hoeffel J, Cleland JL, Daugherty A, van Bruggen N, Redmond HP, Carano RA, Filvaroff EH. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci USA.* 2002;99:9656–9661.
88. Sudmann E, Hagen T. Indomethacin-induced delayed fracture healing. *Arch Orthop Unfallchir.* 1976;85:151–154.
89. Takeda K, Gosiewska A, Peterkofsky B. Similar, but not identical, modulation of expression of extracellular matrix components during in vitro and in vivo aging of human skin fibroblasts. *J Cell Physiol.* 1992;153:450–459.
90. Tencer A, Johnson K. *Biomechanics in Orthopaedic Trauma.* Philadelphia PA: JB Lippincott; 1994:109.
91. Tiyapatanaputi P, Rubery PT, Carmouche J, Schwarz EM, O'Keefe RJ, Zhang X. A novel murine segmental femoral graft model. *J Orthop Res.* 2004;22:1254–1260.
92. Tomford WW, Mankin HJ. Bone banking. Update on methods and materials. *Orthop Clin North Am.* 1999;30:565–570.

93. Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ, Rosen V. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nat Genet.* 2006;38:1424–1429.
94. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol.* 1971;231:232–235.
95. Wheeler DL, Haynie JL, Berrey H, Scarborough M, Enneking W. Biomechanical evaluation of retrieved massive allografts: preliminary results. *Biomed Sci Instrum.* 2001;37:251–256.
96. Whitfield JF. How to grow bone to treat osteoporosis and mend fractures. *Curr Rheumatol Rep.* 2003;5:45–56.
97. Wlodarski KH. Normal and heterotopic periosteum. *Clin Orthop Relat Res.* 1989;241:265–277.
98. Xie C, Reynolds D, Awad H, Rubery PT, Pelled G, Gazit D, Guldberg RE, Schwarz EM, O'Keefe RJ, Zhang X. Structural bone allograft combined with genetically engineered mesenchymal stem cells as a novel platform for bone tissue engineering. *Tissue Eng.* 2007;13:435–445.
99. Yuasa T, Kataoka H, Kinto N, Iwamoto M, Enomoto-Iwamoto M, Iemura S, Ueno N, Shibata Y, Kurosawa H, Yamaguchi A. Sonic hedgehog is involved in osteoblast differentiation by cooperating with BMP-2. *J Cell Physiol.* 2002;193:225–232.
100. Zhang J, Li L. Stem cell niche - Microenvironment and beyond. *J Biol Chem.* 2008;283(15):9499–9503.
101. Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest.* 2002;109:1405–1415.
102. Zhang X, Xie C, Lin AS, Ito H, Awad H, Lieberman JR, Rubery PT, Schwarz EM, O'Keefe RJ, Guldberg RE. Periosteal progenitor cell fate in segmental cortical bone graft transplantations: implications for functional tissue engineering. *J Bone Miner Res.* 2005;20:2124–2137.
103. Zhou Y, Chen F, Ho ST, Woodruff MA, Lim TM, Hutmacher DW. Combined marrow stromal cell-sheet techniques and high-strength biodegradable composite scaffolds for engineered functional bone grafts. *Biomaterials.* 2007;28:814–824.